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13. ABSTRACT ***(Maximum 200 Words)***

Accumulating evidence indicates that BRCA1 is a component of large molecular weight complexes. BARD1-BRCA1 is reported to be one such complex containing BRCA1. To date, there has been no attempt to purify BARD1-containing complexes. Biochemical purification can yield valuable insights into the polypeptide composition and the functional role of mltiprotein complexes. Although the genetic approaches have been successful in defining the genes that are mutated in breast cancer, functional understanding of the protein product of these genes requires biochemical studies. Biochemical analysis of the gene products of BARD1, BRCA1 and BRCA2 will not only reveal their normal cellular function but also indicate the functional defects associated with the mutated proteins. Although such biochemical approaches have not been applied to studies of breast cancer, they have been successfully utilized in understanding complex cellular processes such as transcriptional regulation. We will isolate and functionally define the BARD1-BRCA1-containing complex. We hypothesize that BARD1 plays a role in maintenance of genome stability through its interaction with the BRCA1. We will use biochemical techniques that have been instrumental in increasing our understanding of the transcription machinery, and that have not yet been fully utilized in studies of breast cancer, to isolate the BARD1-BRCA1 complex. We intend to identify BRCA1-associated proteins that are altered as the result of mutations in BRCA1 protein.

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Introduction:

We are using biochemical techniques to define the mutli-protein complexes that contain the breast cancer causing gene BRCA1. These studies are aimed at elucidating the precise biochemical pathways that the BRCA1-BARD1 complex orchestrates. Moreover, we are identifying BRCA1-BARD1-associated genes, whose mutations may underlie the majority of undefined sporadic breast and ovarian cancers.

BODY:

Task 1. Isolate and define the molecular characteristics of the BRCA1-BARD1 complex, using affinity purification by BARD1 antibodies. (months 1-18). This task has been accomplished.

To gain further insight in the molecular mechanism of BRCA1-containing complexes, we generated stable cell lines expressing epitope tagged BARD1, BRCA1 associated RING finger protein. Using these cell lines, we have isolated a multiprotein complex termed BRCC containing BRCA1, BRCA2 and RAD51. Interestingly the E3 ubiquitin ligase activity of BRCC behaves differently than recombinant BRCA1/BARD1 complex. This difference results from the association of the negative regulator BRCC36, a novel component of the complex displaying sequence homology with a 26S proteasome subunit. These results demonstrate the stable association of BRCA2 with a ubiquitin ligase complex that is regulated through a direct interaction with a novel subunit. A full report is being submitted for publication in which the department of defence is credited for their support of our research efforts.

Isolation of BRCC a BRCA1- and BRCA2-containing complex.

To isolate BARD1-containing complex(es) we developed cell lines expressing Flag-tagged BARD1. We were successful in obtaining stable H1299 and 293 cell lines expressing Flag-BARD1. Fig 1A depicts the purification of Flag-BARD1 using anti-Flag antibodies from H1299 cells. An untransfected nuclear extract from H1299 cells were

prepared to serve as the control for anti-Flag affinity purification. Analysis of the Flag-BARD1 eluate using silver staining revealed the specific association of BARD1 with polypeptides of 350, 300 210, 120, 100 (Flag-BARD1), 45, 40 and 36 kDa (Fig. 1A lane 1). A combination of western blotting and mass spectrometric analysis identified the 350, 210 and 40 kDa bands as BRCA2, BRCA1 and RAD51 respectively (Fig. 1A). Analysis of number of preparations indicated that RAD51 corresponds to a substoichiometric component of this complex (also see Fig. 1B). The 300, 120, 45 and 36 kDa polypeptides correspond to novel genes. We therefore termed this complex BRCC for BRCA1/2-containing complex.

To establish that BRCC represent a single complex and is not specific to H1299 cells, we isolated BRCC from a 293-derived cell line expressing Flag-BARD1 and fractionated the complex by cation exchange chromatography (Fig.2 B). As figure 2B indicates BRCA2, BRCA1, BARD1 and RAD51 coelute together peaking in fractions 18 through 20. However, a small fraction of RAD51 dissociates from the complex eluting at fraction 10, consistent with a modular nature of RAD51 association with BRCC. Finally, immunoprecipitation experiments using anti-BRAD1 and anti-BRCA1 antibodies demonstrate the association of BRCA1, BRCA2 and BARD1 from nuclear extract of untagged 293 cells (Fig. 1C). Taken together, these results demonstrate the stable association of BRCA1, BRCA2, BARD1 and RAD51 in a multiprotein complex.

BRCC displays a Ubc5-dependent E3 ubiquitin ligase activity that ubiquitylate P53

Previous reports have pointed to the BRCA1-BARD1 heterodimer as an E3 ubiquitin ligase. We therefore asked whether BRCC display E3 ubiquitin ligase activity and whether its activity behaved similarly to that of recombinant BRCA1-BARD1. Recombinant BRCA1-BARD1 was generated by co-expressing GST-tagged BRCA1(1-639) and Flag-tagged BARD1 as previously descried (Fig. 2A). Analysis of BRCC demonstrated a Ubc5-dependent E3 ligase activity similar to that of recombinant BRCA1-BARD1 complex (Fig. 2B). Ubc5c behaved as the most active E2 with either recombinant BRCA1-BARD1 or the BRCC complex as the E3 enzyme (Fig. 2B). Since a number of previous reports had pointed to a functional and physical association of

BRCA1 and BRCA2 with P53 protein, we asked whether P53 can serve as the substrate for ubiquitination by BRCC. As figure 2C demonstrates P53 can be specifically ubiquitinated by either recombinant BRCA1-BARD1 or by BRCC. These results demonstrate a role for BRCC as a ubiquitin ligase complex that can target P53 for ubiquitylation *in vitro*.

A full report of these studies is being submitted for publication for which department of defence is acknowledged for their support.

Tasks 2 and 3 are being pursued as delineated in the original application.

Figure Legends.

Fig. 1 Purification of the BRCC complex. A) Analysis of anti-FLAG eluate by SDS-PAGE followed by silver staining. Astrics denote non-specifc polypeptides. B) Purification scheme and Wesetrn blot analysis of MonoQ column fraction using antibodies to the right of the figure. I denotes the Flag-eluate which serves as the input for chromatography. C) Immunoprecipitation followed by western blo analysis using antibodies denoted in the figure.

Fig. 2 BRCC is an ubiquitin E3 ligase toward P53 protein. A) Colloidal blue staining of recombinant GST-tagged BRCA1(1-639, G-BRCA1) and full-length FLAG-BARD1 (F-BARD1) coexpressed in bacteria. B) Ubiquitin ligation assay using either recombinant G-BRCA1-F-BARD1 or BRCC as the source of E3. Different E2s are denoted on the top of the figure. C) Ubiquitin ligation assay using Ubc5c as the E2 and P53 as substrate. Recombinant G-BRCA1-F-BARD1 or BRCC was used as the source of E3.

Key Research Accomplishments:

1-Isolated the BRCA1-BARD1 complex from human cells.

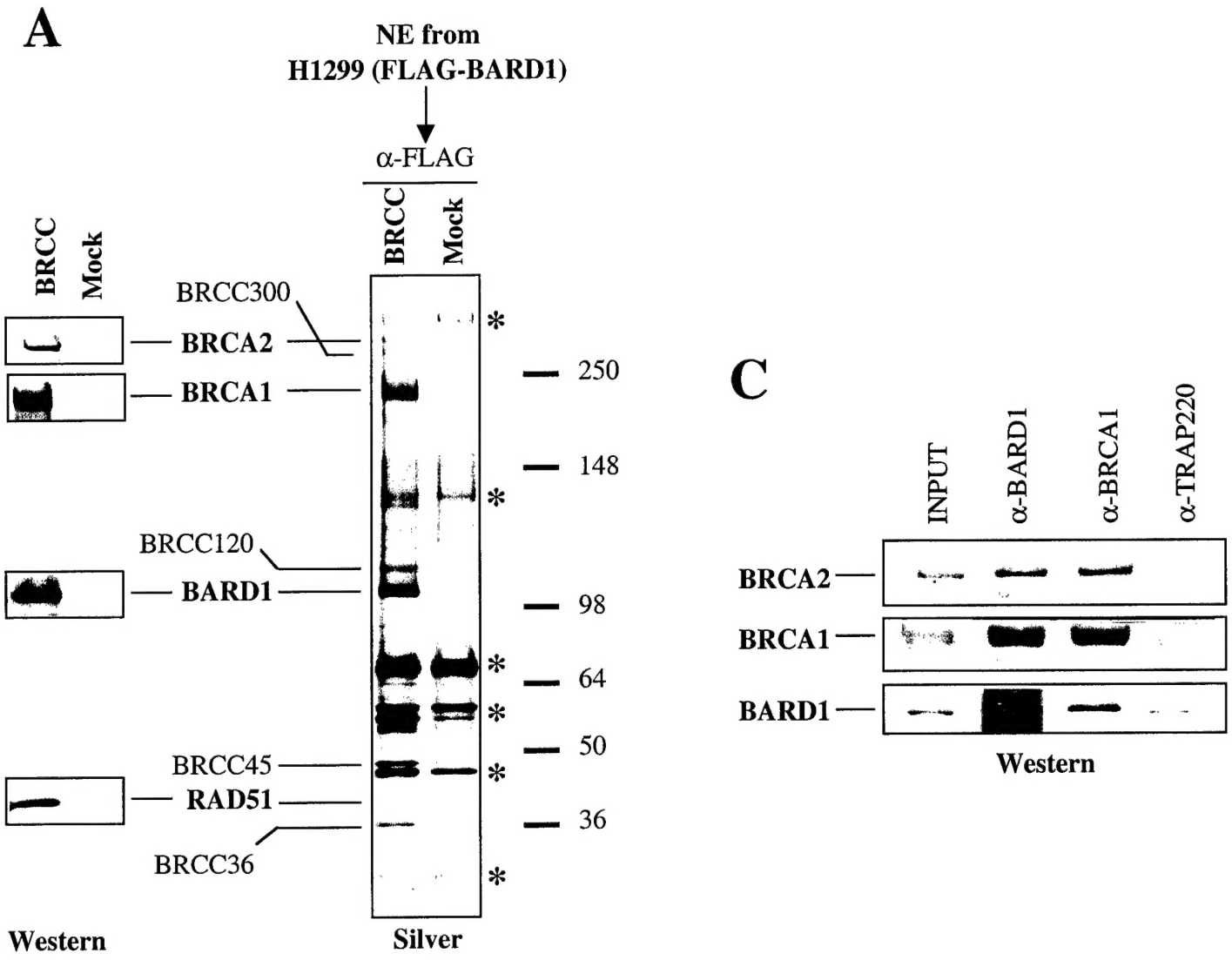
2-Determine the poypeptide composition by MS/MS sequencing.

3-Characterized the ubiquitin ligase activity of BRCA1-BARD1 complex.

Reported Outcomes:

We have a manuscript under revision on the work presented above. Also we are submitting patent applications on the genes that were identified by microsequencing analysis.

Conclusions: We are working toward a complete understanding of molecular events that leads to breast tumor formation. Our goal is to define the biochemical pathway that the breast cancer-causing gene BRCA1 orchestrates. We have isolated the BRCA1-containing complexes from human cells and by characterizing these complexes we will not only gain insight into the molecular mechanism of breast cancer but also identify novel genes whose mutations may result in cancer.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NE from
H1299 (FLAG-BARD1)
α-FLAG
BRCC
Mock
BRCC300
BRCA2
BRCA1
BRCC120
BARD1
BRCC45
RAD51
BRCC36
250
148
98
64
50
36
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Silver
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α-BRCA1
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BRCA1
BARD1
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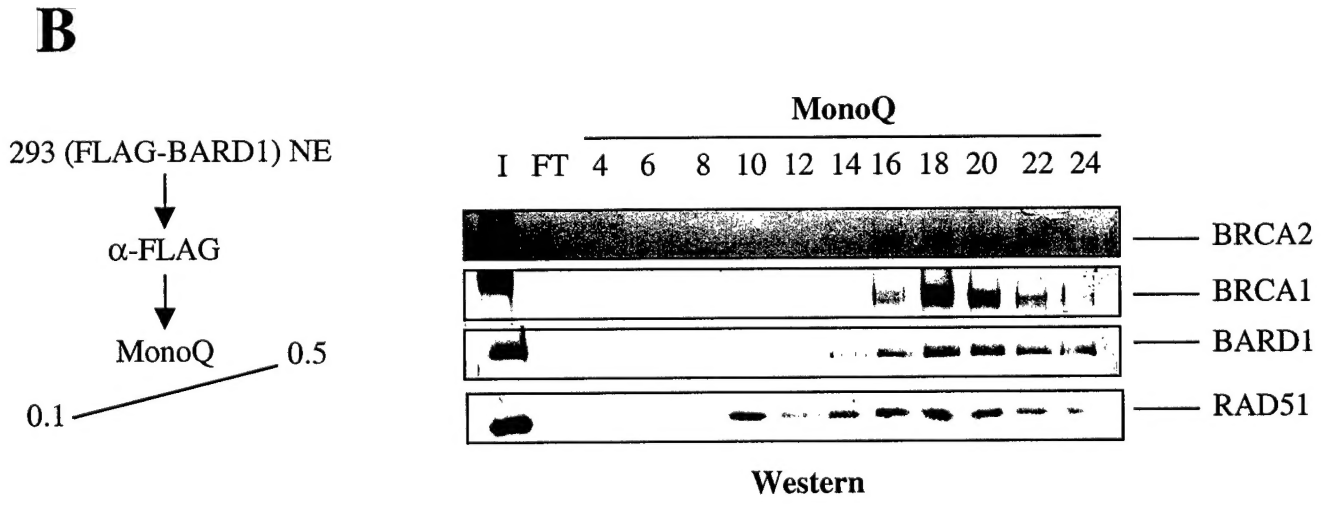
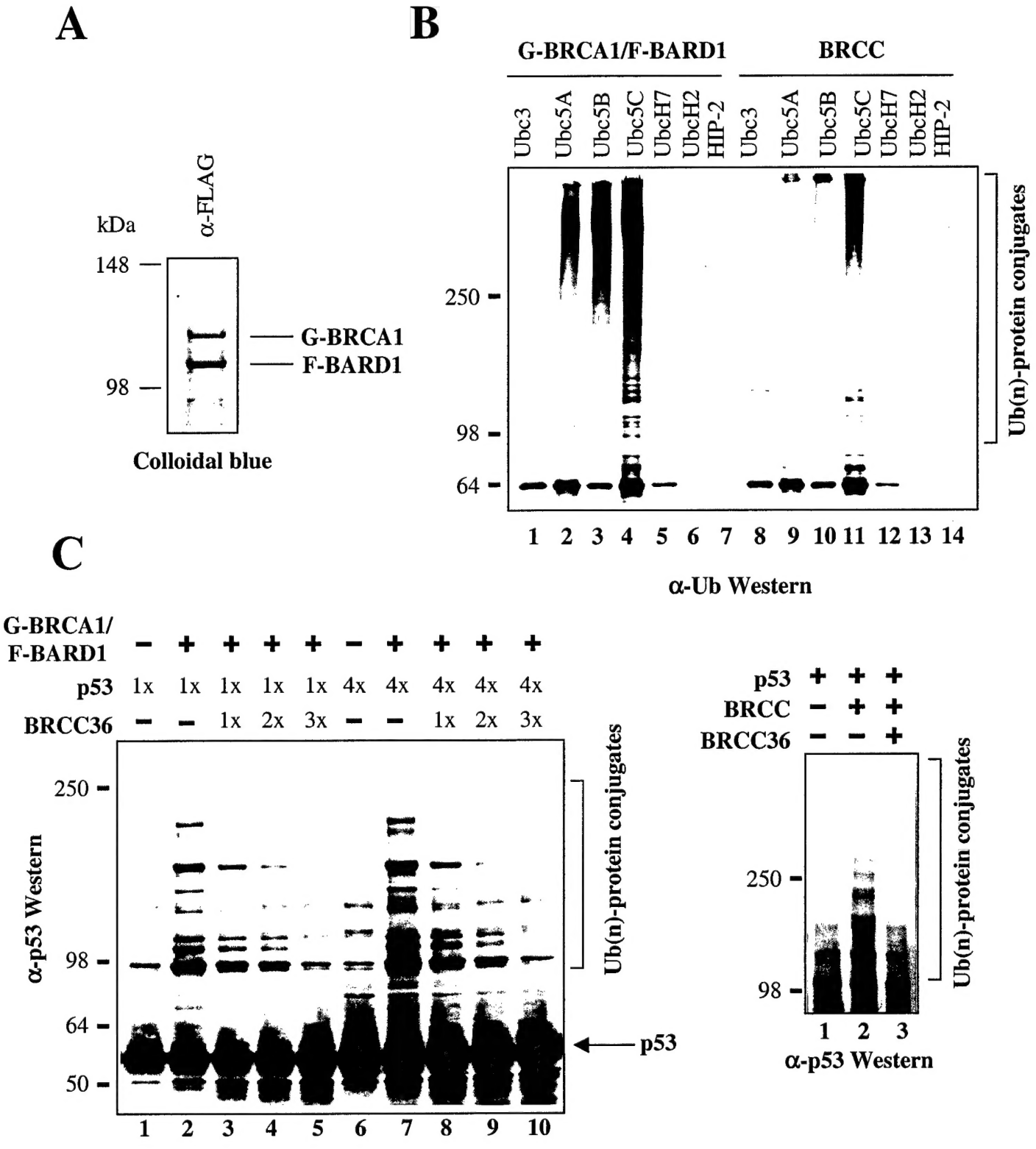

B
293 (FLAG-BARD1) NE
α-FLAG
MonoQ
0.5
0.1
MonoQ
I FT 4 6 8 10 12 14 16 18 20 22 24
BRCA2
BRCA1
BARD1
RAD51
Western

Figure 1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148
98
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F-BRCA1
Colloidal blue
B
G-BRCA1/F-BARD1
BRCC
Ubc3
Ubc5A
Ubc5B
Ubc5C
UbcH7
UbcH2
HIP-2
250
98
64
Ub(n)-protein conjugates
1 2 3 4 5 6 7 8 9 10 11 12 13 14
α-Ub Western
C
G-BRCA1/
F-BARD1
p53
BRCC36
1x 1x 1x 1x 1x 4x 4x 4x 4x 4x
1x 2x 3x 1x 2x 3x
250
98
64
50
α-p53 Western
Ub(n)-protein conjugates
p53
1 2 3 4 5 6 7 8 9 10
BRCC
BRCC36
1 2 3
α-p53 Western

Figure 2